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Quantitative proteomics by SWATH-MS suggest an association between circulating exosomes and maternal metabolic changes in gestational diabetes mellitus

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Abstract

Several factors including placental hormones (PH) released from the human placenta have been associated with the development of insulin resistance and gestational diabetes mellitus (GDM). However, circulating levels of PH do not correlate well with maternal insulin sensitivity across gestation, suggesting that other, previously unrecognized, mechanisms may be involved. The levels of circulating exosomes are higher in GDM compared to normal. GDM derived exosomes produce greater release of pro-inflammatory cytokines from endothelial cells compared to exosomes from normal, suggesting that their contents may differ compared to normal pregnancies. Using a quantitative, information-independent acquisition (Sequential Windowed Acquisition of All Theoretical Mass Spectra [SWATH]) approach, we identify differentially abundance circulating exosome proteins in women with normal glucose tolerance (NGT) and GDM at the time of GDM diagnosis. A total of 78 statistically significant proteins in the relative expression of exosomal proteins in GDM compared with NGT. Bioinformatic analysis showed the exosomal proteins in GDM target pathways were mainly associated with energy production, inflammation, and metabolism. Finally, we used an independent cohort of patients to validate some of the proteins identified by SWATH. The data obtained may be of utility in elucidating the underlying physiological mechanisms associated with insulin resistant in GDM.

Statement of significance of the study

GDM is glucose intolerance that happens during pregnancy and the prevalence is on rise worldwide. Although the condition disappears postpartum, it can have unfavourable long-term effects on mother and child. The lack of understanding of the exact causes of GDM makes it difficult to prevent the incidence of GDM. Thus, understanding the underlying mechanisms of GDM is important for early diagnosis and prevention of GDM. Previously, we reported the concentration of circulating exosomes is higher in GDM compared with healthy pregnancies. In this study, we have characterised the protein content of exosomes present in maternal circulation from GDM and normal pregnancies. We report significant differences in the protein content between GDM and normal pregnancies, with proteins involved in metabolic processes and biological regulation being the most upregulated in GDM. Notably, we identified and validated two proteins involved in the regulation of insulin sensitivity, PAPP-A and CAMK2β, which have previously been reported to be downregulated and upregulated in GDM, respectively. Consistently, in this study we found PAPP-A was downregulated and CAMK2β was upregulated in the exosomes isolated from GDM pregnancies.This new information will help us better understand the function of circulating exosomes in GDM pregnancies, and their potential role in the maternal metabolic adaptation during gestation.

1. Introduction

Gestational diabetes mellitus (GDM) refers to any degree of glucose intolerance first recognised during pregnancy, affecting 5-20% of pregnant women worldwide [1], with a 13% prevalence in Australia [2]. Although the prevalence is increasing, the underlying cellular mechanism(s) in the development of GDM remain unknown.

Recent studies have focused on the potential utility of extracellular vesicles, especially exosomes in monitoring the prognosis and progression of various diseases [3]. Exosomes are small (around 100 nm) vesicles originating from the endocytic pathway that are released to the extracellular milieu by fusion of the multivesicular bodies with the plasma membrane [4]. Exosomes carry a wide range of bioactive molecules including proteins, lipids and nucleic acids, which can be deliver to other cells to facilitates intercellular communications [5]. Exosomes are released from a wide range of cells including the human placenta [6]. Recently, we and others [7, 8, 9] have established the involvement of circulating exosomes in normal and complicated pregnancies (including GDM).

During pregnancy, placenta-derived exosomes present in maternal circulation have been identified as early as 6 weeks of gestation [6]. The release and protein content of exosomes is regulated by factors which include both oxygen tension and glucose concentration and affect their bioactivity on target cells [9-12]. Maternal obesity is associated a higher risk of GDM, and the concentration of placental and non-placental exosomes present in maternal circulation across gestation is influenced by the maternal BMI [13], suggesting that the maternal metabolic state might affect the levels of circulating exosomes. Recently, we established that the total number of exosomes present in maternal plasma was ~2-fold greater in women between 11 to 14 weeks who were subsequently identified as having GDM (diagnosed between 22-28 weeks) than in women who maintained normoglycemia across pregnancy [7].

Taken together, these results suggest that placental exosomes, more numerous in GDM, may play an important role in feto-maternal communication under both normal and pathological conditions. However, the content of the circulating exosomes in GDM has not been established yet.

Several attempt have been made to identify the protein content of exosomes during pregnancy, including exosomes isolated from trophoblast cell lines [14, 15], placental explants [16], placental mesenchymal stem cells [11], primary trophoblast cells [10], endometrial epithelial cells [17], amnion epithelial cells [18] and plasma from pregnant women [6] and umbilical cord blood [19] . A wide range of proteins have been identified in exosomes from placental cells, and circulating exosomes during pregnancy; however, the majority of these studies used standard mass spectrometry discovery approaches, which did not include a quantification of the proteins within the exosomes. Therefore, the aim of this study was to characterise changes in exosomal proteins in plasma from NGT women and women with GDM using a quantitative, data-independent acquisition mass spectrometry approach. For some of the proteins, the results were validated using an independent cohort of patients at the moment of the diagnosis of GDM. Using a bioinformatics approach, we identified signalling pathways associated with the differences in the protein content within exosomes from GDM compared with NGT pregnancies, suggesting that the circulating exosomes in GDM might regulating the maternal metabolic changes associated with insulin resistance in GDM pregnancies. This is the first study using a quantitative proteomic approach to identified the protein profile in circulating exosomes in women at the moment of the diagnosis of GDM (i.e. 22-28 weeks of gestation), thus, the women involved in this study were not under any medication (*i.e.* insulin) or treatment (*i.e.*, diet) to regulate their glucose levels at the moment of the sample collection.

2. Materials and methods

2.1. Regulatory environment and data quality assurance

The project was approved by the Human Research Ethics Committees of the Royal Brisbane and Women's Hospital and the University of Queensland (HREC/09/QRBW/14). Written informed consent was obtained from all women participating in the study. All experimental procedures were conducted within an ISO17025 accredited (National Association of Testing Authorities, Australia) research facility. All data was recorded within a 21 Code of Federal Regulation (CFR) part 11 compliant electronic laboratory notebook (Lab Archives, Carlsbad, CA 92008, USA). Written informed consent was obtained from all women participating in the study.

2.2. Study group and samples

A case control study design was used to evaluate pregnancy-associated changes in exosomal protein in maternal blood obtained from women with normal glucose tolerance (NGT) and GDM pregnancies. Blood samples were collected from study participants between 22-28 weeks of gestation during their diagnostic oral glucose tolerance test (OGTT) for GDM. In this study, we used two independent cohort of patients matched by maternal BMI to identified the proteomic profile in exosomes (i.e discovery phase, with n=11 for NGT and n=11 for GDM), and two selected proteins were validated in an independent cohort of subjects (validation phase, with n=23 NGT women and n=13 women with GDM) by ELISA. Plasma samples were stored at –80°C. GDM was diagnosed by testing with a three sample 75

g OGTT at 24-28 weeks, with cut-offs set according to ADIPS and WHO recommendations [20]. Demographic data of all participants (discovery and validation cohort) involved in this study are summarised in Table 1.

2.3. Isolation and characterisation of exosomes

Exosomes were isolated from plasma as previously described with slight modification [10]. In brief, plasma was diluted with an equal volume of PBS (pH 7.4) and centrifuged at 2,000 x *g* for 30 min at 4 ^{0}C (Sorvall®, high speed microcentrifuge, fixed rotor angle: 90^0, Thermo Fisher Scientific Ins., Asheville, NC, USA,). The 2,000 x *g* supernatant (SN) fluid was then centrifuged at 12,000 x *g* for 45 min at 4 ^{0}C (Sorvall, high speed microcentrifuge, fixed rotor angle: 90^0). The resultant SN fluid (2 ml) was filtered through a 0.22 μm filter (Steritop^{TM}, Millipore, Billerica, MA, USA) and then centrifuged at 100,000 x g for 2 h (Sorvall, T-8100, fixed angle ultracentrifuge rotor). The pellet was suspended in PBS (10 ml) and then transferred to an ultracentrifuge tube (Beckman, 10 ml) and centrifuged at 100,000 x g for 2 h. The 100,000 *g* pellet was resuspended in 300 μl of PBS for size exclusion chromatography (SEC). The SEC was performed using in-house columns. Briefly, Pierce™ Disposable Columns, 10 mL (Thermo Scientific) were packed with 10ml of Sepharose® CL-2B (Sigma) beads and left overnight at 4°C to form a packed bed. The packed bed was equilibrated with ice cold PBS and topped with column filter. The 300 μl of clarified plasma was overlaid on top of the filter and followed by elution with PBS. Five-hundred-microliter of 12 fractions were collected and particle concentration determined using nanoparticles tracking analysis (NAT, NanoSight). High particle fractions were pooled and stored at -80^{o}C until exosome analysis. Exosomes were characterized by size distribution, abundance of proteins associated with exosomes (i.e. CD63, sc15363 [1:1000] and TSG101, EPR7130 [1:1000] and a negative control Grp94, 20292T[1: 1000]) and morphology using Nanoparticle Tracking Analysis

(NTA), Western blot analysis and electron microscopy, respectively as previously described [21].

2.4. Mass spectrometry

2.4.1 In-gel Digestion

A local ion library was generated to use in the Sequential Window Acquisition of All Theoretical (SWATH) mass spectra analysis using an in-gel digestion method. Briefly, two protein pools were prepared from the NGT and GDM plasma exosomes. The samples were mixed with Bolt™ LDS sample buffer (ThermoFisher), sonicated for 5 min and heated at 95°C for 5 min. Samples were resolved on a Bolt™ Bis-Tris Plus polyacrylamide gel (ThermoFisher) at 160 V until full separation. The gel was stained with SimplyBlue™ SafeStain (ThermoFisher Scientific) and a total of 12 gel fractions were excised for each pooled sample. The fractions were washed firstly with 50 mM of ammonium bicarbonate/acetonitrile (ABC/ACN) followed by ACN. 50 µl of 100 mM DTT was added to each samples and incubate at 56°C for 30 minutes. DTT was removed and 70 µl of iodoacetamide (IAA) was added and incubated at room temperature (RT) for 20 min. The samples were washed with 300 µl ACN and incubated with 50 mM ABC/ACN for 30 min at room temperature. Then, 300 µl of ACN was added and left for 2 min. ACN was removed and air dried for 5 min. 50 µl of 13 ng/µl of trypsin (Promega, Australia) in ABC was added to the alkylated gels and stored on ice for 30 min. Then, 20 µl of 50 mM ABC/H_2O (v/v) was added and incubated overnight at 37°C. Following overnight incubation, the supernatant containing peptides was reserved. A mix of 100 µl of extraction buffer (0.25 ml 5% (v/v) formic acid, 0.25 ml water and 0.5 ml ACN) was added to the gel pieces and

sonicated for 10 min. The resulting SN was collected and combined with the reserved SN. The SN were dried in vacuum centrifuge. The dried samples were resuspended in 200 µl 0.1% TFA.

2.4.2 Filter Aided Sample Preparation

For SWATH analysis, individual exosome samples were processed using the Filter Aided Sample Preparation (FASP) method [22]. A total of 15 µg of exosome protein from each sample was reduced with equal volume of lysis buffer containing 8% SDS, 100 mM Tris, pH 7.6 and 0.2 M DTT, followed sonication and heating of samples at 95°C, each. Samples were allowed to cool down completely before adding 8 M urea in 100 mM Tris, pH 8.5. Samples were transferred into a Nanosep® filter unit with a 30K molecular weight cut off and centrifuged for 10,000 *g* for 15 min. Then, filter units were washed with 400 µl of urea buffer and centrifuged for 10,000 *g* for 15 min. Samples were alkylated by addition of 100 µl of 50mM IAA in 8M urea buffer and incubated in the dark for 20 min. The filter units were washed with 8M urea buffer followed by of ABC. Proteins were digested using 0.3 µg of trypsin and incubated overnight at 37°C.

2.4.3. Desalting

The solubilised peptides from pooled and individual samples were desalted using SOLAµ HRP SPE 96 well plate (Thermo Fisher Scientific) according to manufacturer's instruction.

2.4.4. Analysis of peptides

Tryptic digest was loaded onto a reversed phase trap column (CHROMXP C18CL 5um, 10 x 0.3mm; Eksigent, Redwood City) and on column wash was performed for 15 min (3 ul/min) followed by peptide separation on reversed phase CHROMXP C18CL 3 um, 120 A^0, 150 x 0.075mm; (Eksigent, Redwood City) analytical column. LC gradient started with 95% mobile

phase A (H_2O/ 0.1% FA), 5% B (ACN/ 0.1% FA) at 0 min and increase to 10% B over for 2 min and then a 58-min linear gradient to 40% B followed by 50% B for 5 min. Mobile phase B was then increased from 50% to 95 % over 10 min followed by column wash at 95% B for 15 min and re-equilibrated with 5% Buffer B for 6 min. Flow rate was kept at 250 nl/min during entire LC run. The resulting peptide samples were processed in IDA on an AB Sciex 5600 TripleTOF mass spectrometer with the top 18 precursor ions automatically selected for fragmentation. The data obtained were combined to establish a peptide ion database. For SWATH acquisition, the TripleTOF® 5600 System was configured as described by Gillet at al. [23]. Using an isolation width of 26 Da (25 Da of optimal ion transmission efficiency and 1 Da for the window overlap), a set of 32 overlapping windows was constructed covering the mass range 400 to 1200 m/z.

2.4.5. Data Processing

To generate the local ion library, a protein database search was conducted using the ProteinPilot version 4.5b Software (AB SCIEX) and the Paragon™ Algorithm. The search was performed against SwissProt Homo sapiens database with a global false discovery rate (FDR) of 1% was used as the threshold for the number of proteins for import. The SWATH Acquisition Microapp 2.0 in PeakView 2.2 (SCIEX) was used to create a spectral library file. This local library was extended using the R package SwathXtend (version 2.3) [24] with a published SWATH dataset of healthy human plasma [25]. The extended library was used for all subsequent SWATH analysis. Processing settings for the SWATH Microapp: 2 peptides per protein, 3 transitions per peptide, peptide confidence threshold corresponding to 1% global FDR and FDR threshold of 1% was used. The retention time was then manually realigned with a minimum of 5 peptides with constantly high signal intensities and distributed along the time axis. The resulting peak area for each protein after SWATH processing was exported to MarkerView 1.3.1 (SCIEX) for statistical analysis. The resulting data was

normalized using the Total Area Sums (TAS) approach. The coefficient of variation in the abundance of peptides across the samples were established by comparing SWATH peptide ion against the IDA library. For independent sample, *t*-tests were used to compare protein expression between NGT and GDM groups. The proteins with $p<0.05$ were considered as statistically significant.

2.5. Validation of SWATH data using an independent cohort of patients.

The differentially expressed candidate proteins obtained from the SWATH analysis were validated on an independent cohort of patients using an enzyme-linked immunosorbent assay (ELISA). Quantitative measurements of calcium/calmodulin dependent protein kinase II beta (CAMK2β: ab234572) and Pappalysin-1 (PAPP-A: ab235647) were performed according to manufacturer's instruction (Abcam, Cambridge, UK), with minor changes. To ensure the exosomes were lysed before the commencement of the assay, samples were diluted in the provided cell extraction buffer and sonicated for 10 mins (Elma ultrasonic, Sinden, Germany).

2.6. Bioinformatics analysis

The origin of the exosomes present in maternal circulation was identified using FunRich [26], an open access standalone functional enrichment and interaction network analysis tool. Differentially expressed proteins were analyzed further by bioinformatic pathway analysis (Ingenuity Pathway Analysis [IPA]; Ingenuity Systems, Mountain View, CA; www.ingenuity.com).

3. Results

3.1. Clinical characteristics of the study population

In total sixty-one women (NGT = 37, and GDM = 24) were involved in this study. Analysis if the clinical characteristics of the women showed that no difference in maternal age, BMI at pre-pregnancy and at delivery, gestational age at delivery and fetal birth weight between women with NGT women and GDM were identified (Table 1). As expected, women in the -GDM group had significantly higher glucose levels at all time points of the OGTT, which is the criteria of diagnosis of GDM. Next, exosomes were isolated from plasma obtained from these women and characterised.

3.2. Exosomes characterisation

The flow diagram of exosome isolation and enrichment from plasma is presented in Figure1A. The NTA analysis identified vesicles with a diameter between 50 to 150nm, with an enrichment of vesicles of 100 nm (Figure1B). Exosomes were positive for proteins known to enriched in exosomes, *i.e.,* CD63, CD9 and TSG101 while negative for Grp94 an endoplasmic reticulum marker (Figure 1C). This demonstrates the purity of the isolated exosomes. There were no differences in exosome size distribution and abundance of exosome-associated protein markers between exosomes isolated from NGT and GDM plasma, indicating that GDM does not impact upon the size distribution of exosomes.

3.3. Proteomics analysis and associated signalling pathways

Information-dependent acquisition (IDA) and SWATH profile were generated from NGT and GDM using independent samples (n=11) per each group (*i.e.* NGT or GDM). The IDA library was used to identify peptide ions that were present in SWATH ion profiles. Proteins were identified and quantified by comparing SWATH-generated peptide ion profiles for each individual sample against the IDA library (PeakView). IDA of mass spectra from all

individual exosomes samples was initially performed and identified 415 total proteins (Table S1), and analysed using IDA and SWATH. The variation in the relative abundance of exosomal proteins between NGT and GDM was established by comparison with the SWATH profile against the IDA library and presented as volcano plot (Figure 2A). A total of 78 statistically significant protein identifications ($p < 0.05$) in the relative expression of exosomal proteins in GDM compared with normal pregnancy were identified (Table S2). The proteins significantly different (*i.e.* down and upregulated proteins) in exosomes from GDM compared with NGT were subjected to ontology and pathway analysis using Panther and Gene Ontology algorithms and classified based on biological process (Figure 2B and C). The biggest differences for the upregulated compared with the downregulated proteins in the clusters identified were in metabolic process (*i.e.* 45% vs 26.5%) and biological regulation (*i.e.* 12.5% vs 5.9%). Then, we sought to characterise the total population of exosomes in maternal circulation with NGT and GDM by characterising the protein composition of plasma exosomes subpopulations. Clustering of the proteins based on their site of expression showed the exosomes from both groups are mainly originated from placenta, CD4, platelets, amniotic fluid, preadipocytes, fetus, erythrocytes, macrophages and adipocytes (Figure 2D). The comparison analysis demonstrated the enrichment of proteins associated with placenta, adipocytes, macrophages, platelets, amniotic fluid, CD4 and fetus while depletion of proteins associated with erythrocytes and preadipocytes.

Next, to investigate the potential functions of these differentially expressed proteins, Ingenuity pathways analysis (IPA) of the exosomal proteomic profile in NGT and GDM was performed Four major networks were identified using the fold changes and p-value of the protein profile in GDM compared to NGT (Figure 3, Table S3). The network included molecules with functions related to Energy Production, Nucleic Acid Metabolism, Small Molecule Biochemistry (Figure 3A), Developmental Disorder, Hematological Disease,

Hereditary Disorder (Figure 3B), Cell-To-Cell Signalling and Interaction, Inflammatory Disease, Inflammatory Response (Figure 3C), and Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry (Figure 3D).

3.4 Validation using an independent cohort

The proteomic analysis showed that two of the proteins within exosomes that were significantly difference between NGT compared to GDM were calcium/calmodulin dependent protein kinase II beta (CAMK2β) and Pappalysin-1 (PAPP-A). Interestingly, CAMK2β was upregulated in exosomes obtained from GDM compared with exosomes from NGT. On the other hand, PAPP-A was downregulated in GDM exosomes compared with exosomes from NGT. Low levels of circulating PAPP-A in maternal plasma has been associated with GDM [27] and high levels of CAMK2 in skeletal muscle from GDM have been associated decrease in insulin sensitive [28]). Therefore, PAPP-A and CAMK2β were chosen for the validation of the proteomic data in an independent cohort of women with NGT and GDM using ELISA kits. PAPP-A was significantly lower (54 ± 25 %) in exosomes obtained from GDM compared with exosomes from NGT (Figure 4A). The concentration of CAMK2β within exosomes was significantly higher in GDM (2-fold) compared to NGT exosomes (Figure 4B). Interestingly, a negative correlation ($p = 0.0135$) between maternal BMI and the concentration of exosomal PAPP-A, and effect that is influence by the pregnancy condition (i.e. NGT or GDM) (Figure 4C). No significant correlation ($p = 0.1859$) between maternal BMI and the concentration of exosomal CAMK2β was identified (Figure 4D).

4. Discussion

Exosomes play a role in intercellular communication during pregnancy. Recently, we have established the presence of higher concentration of exosomes in pregnant women compared non-pregnant and the concentration increases with various pathologic conditions [7, 15, 29]. While circulating exosomes during pregnancy have been previously characterised and quantified [6, 30]; however, no studies have examined differences in protein profile in circulating exosomes between NGT and GDM pregnancies. In this study, we used a cohort of samples obtained at the time of GDM diagnosis (*i.e.,* samples were collected at the time of screening for GDM by OGTT) and employed mass spectrometric analysis to generate proteomic profile of encompassing information on protein abundances in plasma exosomes from NGT and GDM. A total of 415 proteins were detected with 78 proteins significantly and differentially expressed in plasma GDM exosomes compared with NGT. Our data demonstrated in comparison with NGT, circulating exosomes from GDM have differential expression of proteins, including spectrin alpha erythrocytic (SPTA)-1, CAMK2β, PAPP-A, Perilipin 4, fatty acid binding protein (FABP) 4, hexokinase-3. Interestingly, these proteins have been previously shown to be differently expressed in insulin resistance [31]. Using an independent cohort of samples, we validated the differences observed in the protein abundance of PAPP-A and CAMK2β within exosomes isolated from NGT compared with GDM by ELISA.

PAPP-A is a glycoprotein synthesised primarily by the villous and extravillous cytotrophoblasts [32]. ~~PAPP-A possess proteolytic activity towards the insulin-like growth factor binding protein (IGFBP) 4 [33].~~ The concentration of PAPP-A in maternal circulation increases throughout pregnancy and declines postnatally [34]. Low maternal circulating concentrations of PAPP-A is correlated with adverse pregnancy outcomes [35] including GDM [27]. Notably, PAPP-A levels in the first trimester is associated with insulin resistance later in pregnancy [27, 36]. Likewise, in this study we found that PAPP-A expression is lower in GDM

exosomes compared to NGT exosomes. The low expression of PAPP-A in exosomes could have important implication in intercellular communication between placenta and maternal environment which mediates changes in insulin sensitivity.

CAMK2β regulates a range of processes, including metabolism and insulin sensitivity [37]. CAMK2 is a serine/threonine specific protein kinase and acts as an important mediator of calcium homeostasis in cells [38]. Supporting our proteomics results, the quantification of CAMK2β by ELISA in an independent cohort of samples showed higher levels of CAMK2β in GDM exosomes compared with NGT exosomes. These findings are in line with previous study showing upregulation of CAMK2α in skeletal muscle of pregnant women with GDM [28]. Further research on the role of CAMK2β in the pathophysiology of GDM is warranted.

Interestingly, in our study, the GO analysis showed the differently regulated proteins in GDM are highly associated with metabolic processes and biological regulation; Akt signalling and cholesterol were among the target signalling molecule identified by IPA. One of the downstream targets of Akt is mTOR which has been shown to increase placental glucose and lipid metabolism and inflammation in GDM [39]. Collectively, this data suggests that the proteins in GDM exosomes may play a role in aggravating inflammation, activating the placental nutrient centre and contributing to greater transplacental lipid transport and fetal overgrowth.

The comparative analysis of the subpopulations of exosomes showed that proteins from placental tissues were upregulated in GDM exosomes, which is line with our studies demonstrating greater numbers of placental derived exosomes in women with GDM across gestation [8]. Together, these finding suggest increased secretion of placental derived exosomes in GDM pregnancy. Similarly, we found that proteins associated with amniotic fluid and the fetus were greater in GDM plasma exosomes. Although previous studies have

reported on the association between amniotic fluid exosomes and number of pregnancies [40], no study has reported on the association between amniotic fluid exosomes and GDM pregnancies. The observed increased in aminiotic fluid exosomes could be attributed to polyhydramnios [41]. Exosomes from the fetal side has been shown to have the ability to traffic across the placenta and enter maternal circulation [42]. Additionally, large for gestational age and macrosomia are common perinatal outcomes in GDM pregnancies [43]. Based on our results, it is possible the increased fetal associated proteins relates to an increase in fetal exosomes secretion and is associated with fetal overgrowth. Interestingly, we found an enrichment in proteins associated with adipocytes and macrophages but depletion of proteins associated with preadipocytes. Exaggerated adipose tissue dysfunction including hypertrophic expansion and chronic inflammation have been reported in insulin resistance [44]. Inflammation leads to macrophage infiltration in adipose tissue leading to an inability to generate adipocytes and increased storage of lipids. Ultimately, this leads to dysfunctional and necrotic adipocytes [44] characterised by increased adipocytokine release and reduced insulin sensitivity. Based on this, the increased number of proteins associated with adipocytes and macrophages in GDM exosomes may reflect the compromised function of adipose tissue. For example release of high numbers of exosomes in response to oxidative stress and activation of inflammatory pathways by the macrophages. It is possible the interaction between exosomes from maternal circulation and placental cells or any other metabolically active tissues, such as adipose tissue, skeletal muscle or pancreas affecting insulin sensitivity, glucose metabolism and mitochondrial dysfunction by reactive oxygen species production all which are commonly seen in GDM pregnancies [45].

Given that exosomes are highly involved in intercellular communication, the differential abundance of subpopulation of exosomes in maternal circulation may influence the placental

microenvironment and metabolism contributing to development of GDM and its complications, such as fetal macrosomia.

4.1. Conclusions

To the best of our knowledge, this is the first study that profiled the protein content of circulating exosomes in plasma and characterised the subpopulations of exosomes in maternal circulating based on their protein expression. In this study, we have established that GDM is associated with changes in the expression of plasma exosomal proteins. In particular, we have identified 78 proteins which are differently expressed in GDM exosomes, where these proteins are targeting molecules that may relate to GDM pathophysiology. We found that in GDM there is a selective enrichment and depletion of exosomes originating from various sites including placenta, adipocytes, macrophages and preadipocytes. It is important to note that in this study we have isolated and characterised the total population of exosomes present in maternal circulation, and specific isolation of origin-specific exosomes (*e.g.* from placenta) will be required for further studies. The specific isolation of placental exosomes from maternal circulation is challenging due to the levels of placental exosomes present in maternal circulation (around 15% [13] and highly dependent on the specificity of the PLAP antibody. Overall, in this study we report changes in the proteomic profile in the exosomes present in maternal circulation at the moment of the GDM diagnosis compared to women with NGT. Further studies are required to determine the function of circulating exosomes in GDM pregnancies.

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Conflict of interest

The authors have nothing to declare.

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Figure legends

Figure 1: Characterisation of exosomes isolated from NGT and GDM plasma. Exosomes were isolated from maternal plasma by differential and ultracentrifugation followed by size exclusion chromatography. (A) Flow chart for the exosome isolation and enrichment procedure (B) Representative size distribution of exosomes in NTA (C) Representative Western blot for exosome enriched marker CD63, TSG101, CD9 and negative marker, Grp94.

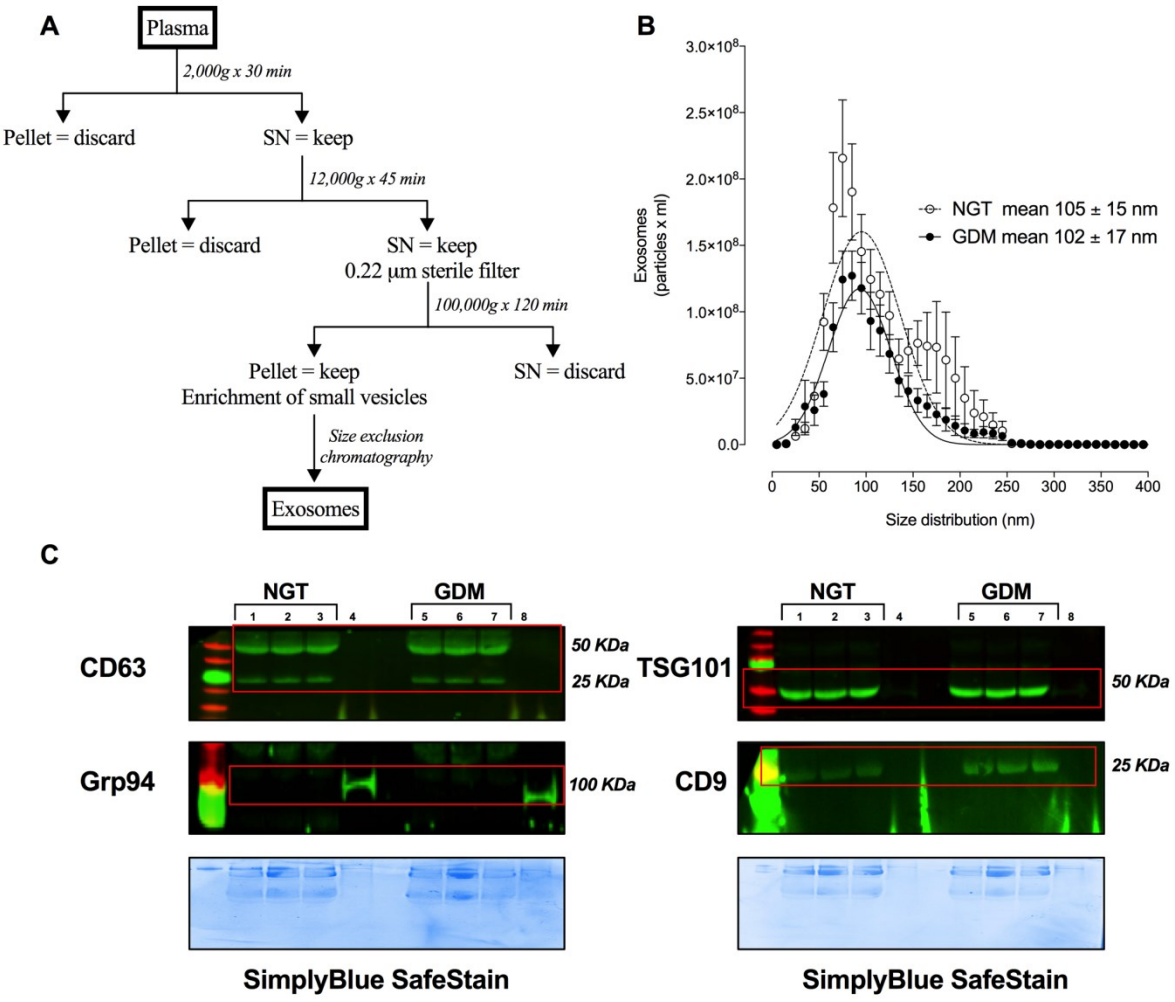

Figure 1

Figure 2: Comparison of protein enrichment in NGT and GDM plasma exosomes. (A) Volcano plot showing differentially expressed protein in the GDM plasma exosomes compared to NGT plasma exosomes. The horizontal axis represents the $\log_2$ of fold change and the vertical axis represent *p-value*. The horizontal dotted line shows p= 0.05. Each black dot represents a protein with black dots on the right above the dashed line are proteins upregulated while on the left are downregulated in GDM exosomes. The gene ontology classification of (B) downregulated and (C) upregulated, on the basis of their involvement in biological process using Panther and Gene Ontology algorithms. (D) Proteins that are enriched and depleted in GDM exosomes compared to NGT exosomes are displayed based on their site of expression using FunRich. The vertical axis represents the site of expression and $\log_2$ of fold change.

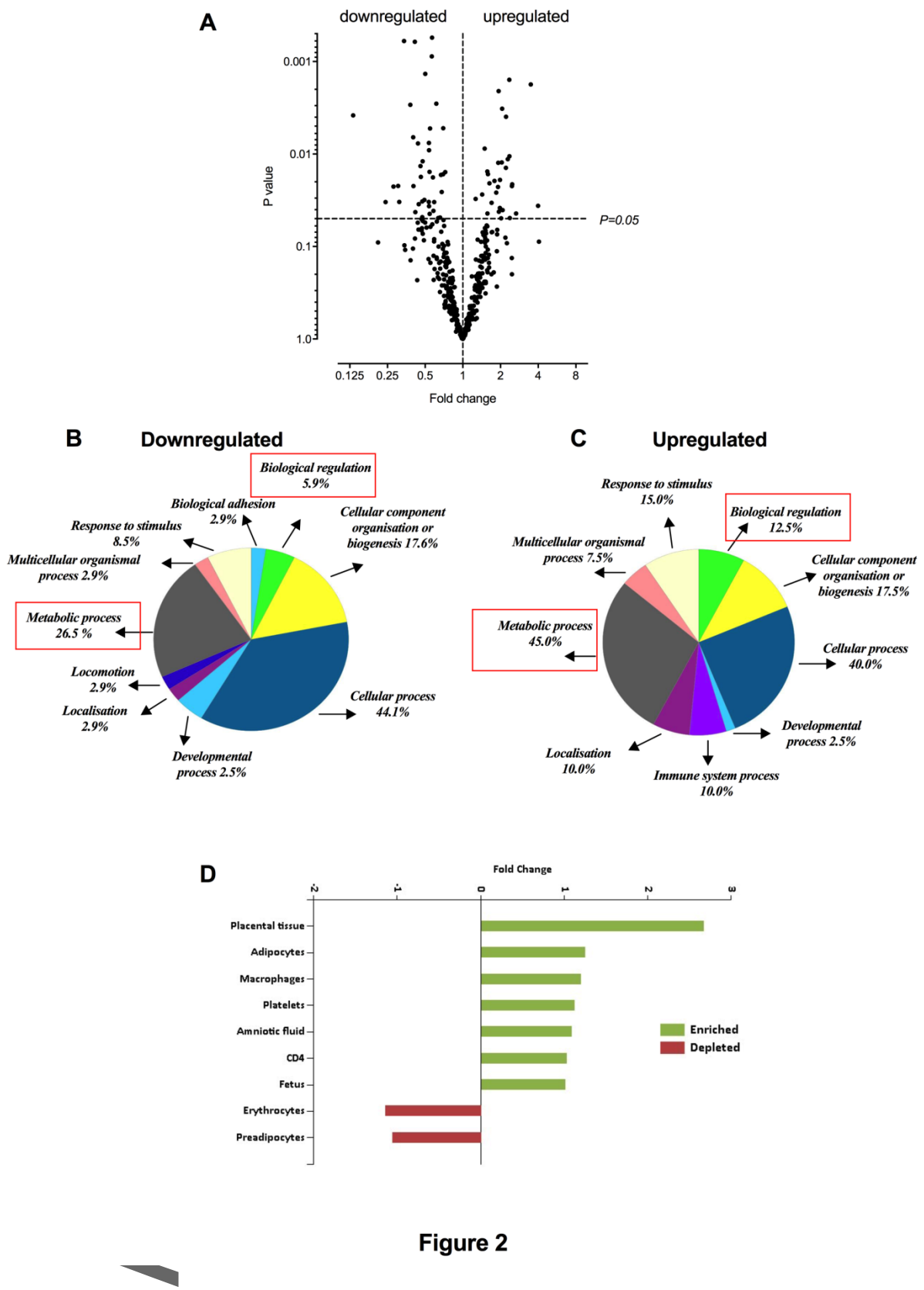

Figure 2

Figure 3: Top 4 networks identified by Ingenuity pathways analysis (IPA). Differently expressed proteins in GDM exosomes were submitted to IPA network analysis. The network represents (A) Energy Production, Nucleic Acid Metabolism, Small Molecule Biochemistry with a score of 56, (B) Developmental Disorder, Hematological Disease, Hereditary Disorder with a score of 30, (C) Cell-To-Cell Signalling and Interaction, Inflammatory Disease, Inflammatory Response with a score of 30, and (D) Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry with a score of 10. Each network displays the genes as nodes and the relationships between the nodes as lines. The colour intensity of each node indicates the degree of upregulation (red) or downregulation (green) of the respective gene transcript.

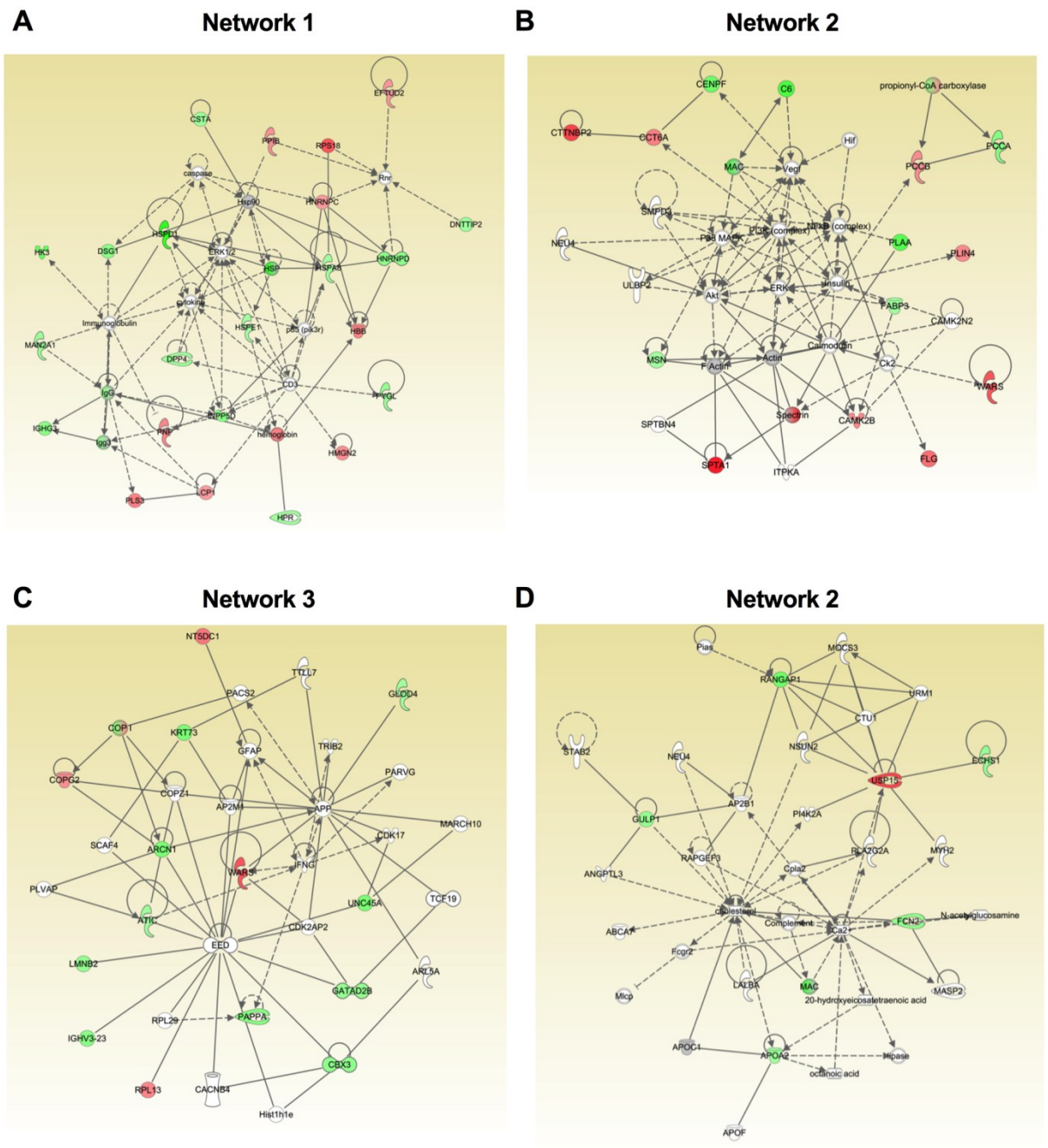

Figure 3

A

Figure 4. Quantification of PAPP-A and CAMK2β using an independent cohort of patients. The concentration (presented as pg of protein per exosome) of PAPP-A and CAMK2β was quantified in exosomes isolated from NGT and GDM by ELISA. (A) Concentration of PAPP-A. (B) Concentration of CAMK2β. (C) Association between the concentration of PAPP-A and maternal BMI. (D) Association between the concentration of CAMK2β and maternal BMI. Data represents n=26 and n=13 for NGT and GDM, respectively. Values are mean ± SD as scatter dot plot.

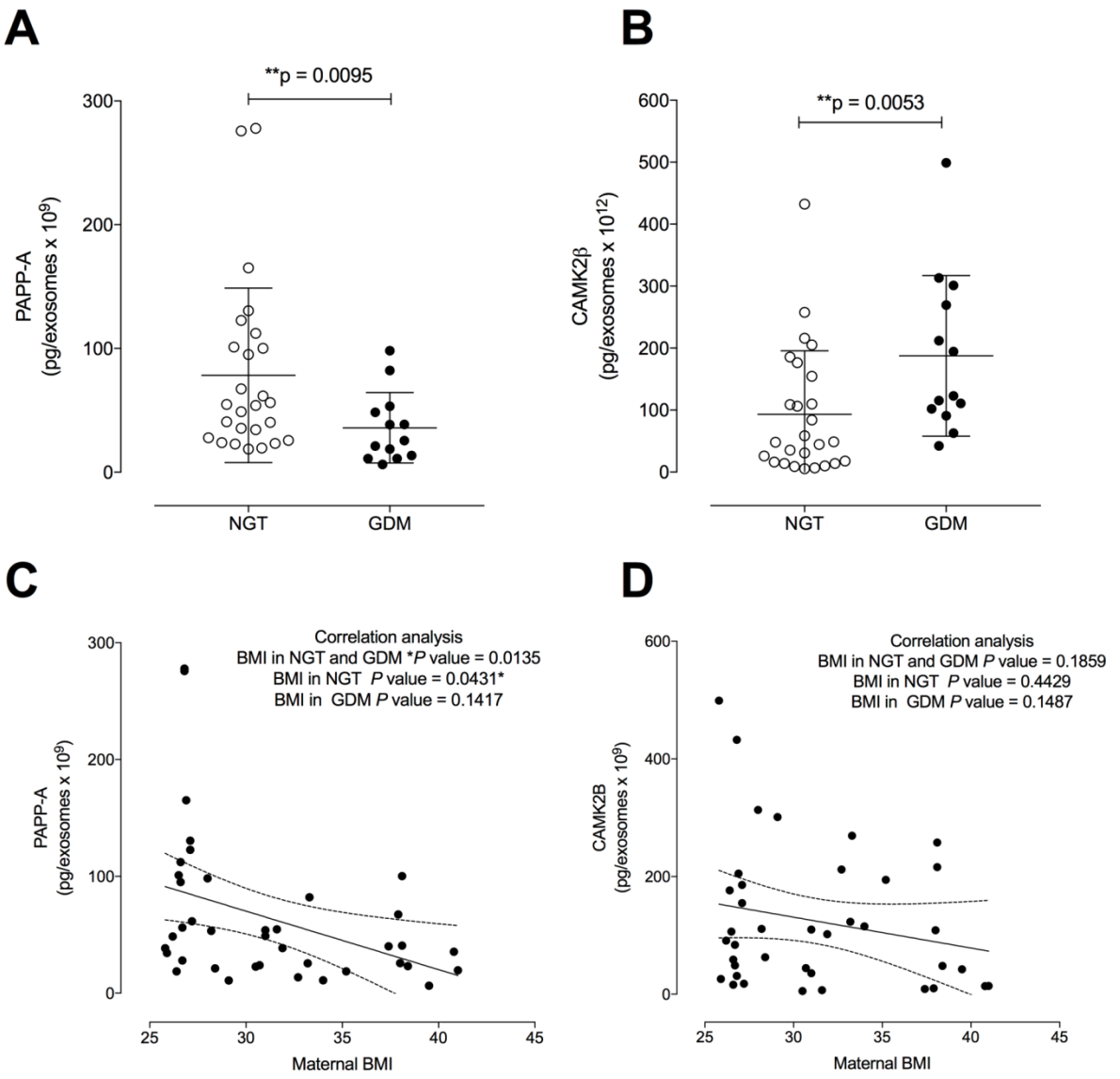

Figure 4

Table 1. Clinical characteristics of groups used for LC-MS/MS discovery and validation phases of this study.

	Discovery cohort			Validation cohort		
Maternal variables	**NGT (n= 11)**	**GDM (n= 11)**	***p-value***	**NGT (n= 26)**	**GDM (n= 13)**	***p-value***
Maternal age (years)	34.12 ± 3.55 (28.11-40.13)	31.27 ± 3.07 (27.23-38.12)	0.0682	30.81 ± 3.08 (25-36)	32.85 ± 4.18 (25-40)	0.0928
Maternal height (cm)	162.11 ± 7.04 (151- 176)	161.42 ± 6.20 (148- 167)	0.0799	163.2 ± 7.62 (146.7-176.4)	164.9 ± 7.44 (152.1-158.3)	0.5096
Weight at pre-pregnancy (kg)	90.89 ± 24.05 (63- 143)	90.21 ± 30.74 (59- 158)	0.9544	83.49 ± 17.59 (58.5 -126.1)	84.02 ± 13.64 (62.0 - 126.1)	0.9247
BMI at pre-pregnancy (kg/ m^2)	34.02 ± 10.02 (25.71-56.31)	34.23± 10.27 (253.8- 58)	0.9619	31.22 ± 5.39 (25.9- 41.0)	31.19 ± 3.97 (25.8-39.5)	0.9856
Weight at delivery (kg)	94.01 ± 21.2 (75 - 143)	101.5 ± 32.24 (70 - 161)	0.546	92.36 ± 17.36 (69.3 -129.1)	91.86 ± 15.24 (71.0 - 116.7)	0.9338
BMI at delivery (kg/ m^2)	36.89 ± 10.65 (28.61-61.51)	38.33 ± 9.53 (29.1-55.1)	0.7537	34.08 ± 4.92 (25.7- 36.7)	33.45 ± 4.44 (28.0-43.5)	0.6978
Fasting glucose (mmol/l)	4.34 ± 0.34 (3.82- 4.92)	5.71 ± 1.20 (4.33-	*0.0015***	4.58 ± 0.30 (4.0-5.0)	5.21 ± 0.63	*0.0001****

		8.71)			(4.3- 6.6)	
OGTT 1 hr glucose (mmol/l)	6.78 ± 1.20 (4.82- 8.71)	12.23 ± 3.15 (8.32- 19.11)	*<0.0001***	6.69 ± 1.49 (3.6-9.4)	10.51 ± 1.90 (7.8- 14.5)	*0.0001****
OGTT 2 hr glucose (mmol/l)	5.87 ± 1.13 (3.61- 7.30)	11.07 ± 4.57 (6.61- 21.12)	*0.0015***	5.83 ± 1.17 (3.2- 7.6)	8.8 ± 2.09 (5.2- 13.3)	*0.0001****
GA at delivery (week)	39.21 ± 1.64 (35.23- 41.21)	38.07 ± 0.90 (36.51- 39.30)	0.0565	39.37 ± 0.97 (37- 41.2)	38.38 ± 0.92 (37- 40.2)	*0.06823*
Fetal variables						
Birth weight (g)	3322.12 ± 596.12 (1938.11- 4080.13)	3357 ± 415.9 (2640- 4070)	0.8738	3300 ± 488.2 (1938- 4200)	3412 ± 647.2 (2440- 4660)	0.548
Fetal gender (M/F)	5/6	6/5	-	9/ 17	7/ 6	-

Data are presented as mean ± SD (range). All pregnancies were normotensive, non-smoking, non-alcohol or drug consuming, and without intrauterine infection or any other medical or obstetrical complications except GDM. NGT: women with normal glucose tolerance test; OGTT: glucose measure was 2-hrs post-glucose challenge (75 g); M = male; F = female; GA = gestational age. For 2-group analyses, Student's tests were used to assess statistical difference. *p<0.05, **p<0.005, and ***p<0.0005 for GDM vs NGT.